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13. SUPPLEMENTARY NOTES

14. ABSTRACT
The primary goal of this collaborative research project is to develop next generation engineered nerve guide conduits (NGCs) with aligned nanofibers and favorable release kinetics of neurotrophic factors to help improve surgical outcomes after injuries involving peripheral nerves.
In the third year of the work, we further developed the NGCs and tested various combinations of NGCs in a rat sciatic nerve regeneration model. We have also obtained the IACUC and ACURO approval for the large animal validation study using the peroneal nerve repair in dogs and started the study. We plan to complete it during the no-cost extension (4th year).

15. SUBJECT TERMS
Nanofiber nerve guides, nerve regeneration, neurotrophic factor, gradient loading

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Introduction:
Peripheral nerve injury is a common complication of complex tissue trauma and often results in significant disability in war injuries. Regeneration of peripheral nerves is often incomplete and in complex war injuries donor nerves are difficult to find for nerve repair. Nerve guide conduits (NGCs) made of biodegradable materials offer a potential solution to this problem. Based on our previous accomplishments in developing a nanofiber containing NGCs, the primary goal of this collaborative research project is to develop new nanofiber NGCs with improved nanofiber guidance cue and modulated trophic factor delivery capabilities that promise faster nerve regeneration and better functional recovery.

Body:

Statement of Work (Proposed Tasks)

The goals of Year 2 included optimization of nanofiber nerve guide design to provide i) contact guidance cue (nanofiber diameter, degradation rate, and fiber density/distribution) and ii) modulated neurotrophic factor delivery (factor choice, concentration range and gradient configuration) for regenerating axons and Schwann cells, and assessing the effects of each modification in a rat model of nerve regeneration. Parts of these goals were not completed in Year 2 but were completed in Year 3. Furthermore, in Year 3 we had planned to carry out Task 3, large animal validation study, but this is largely delayed into the 4th year (a no-cost extension was granted earlier) because of the delays in manufacturing and testing the optimum configuration of the NGCs.

Goals for Year 2 and 3 were outlined in the original Statement of Work as:

Task 2: To assess nerve regeneration rate and functional recovery in a rat model of nerve repair, and to optimize nerve guide configurations

2a. To manufacture of nanofiber nerve guides of without neurotrophic factor loading
2b. To evaluate the effect of nanofiber diameter and degradation rate on nerve regeneration in the rat sciatic model
2c. To manufacture nanofiber nerve guides with optimum neurotrophic factor loading
2d. To evaluate the effect of different neurotrophic factors on nerve regeneration in the rat sciatic model
2e. To prepare and obtain regulatory approval for the dog studies

Task 3: To demonstrate efficacy in a large animal model using optimized nerve guide from Aim 2, as a prerequisite for clinical translation

3a. To manufacture the nanofiber nerve guides to be used in the dog peroneal nerve repair model (months 23-24)
3b. Surgical repair of the peroneal nerve using the nanofiber nerve guides; total 60 dogs used (months 24-25)
3c. Longitudinal gait evaluation for foot drop (months 24-33)
3d. Retrograde labeling and tissue harvesting (months 32-33)
3e. Nerve morphometry (months 33-35)
3f. Histopathological evaluations (months 33-35)

Progress

Specific experiments were completed in preparation for the canine study. Enhancements to the *in vitro* migration analysis platform allowed for high throughput analysis of Schwann cell migration in response to fiber-mediated topographical guidance and neurotrophic factor gradient-mediated chemotrophic guidance. Improvements in our *in vitro* platform as well as improved design of the nerve conduit allowed for significant progress to be made on optimizing nerve conduits incorporating both

nano/microfiber-mediated contact guidance and controlled release of neurotrophic factor gradients (Fig. 1).

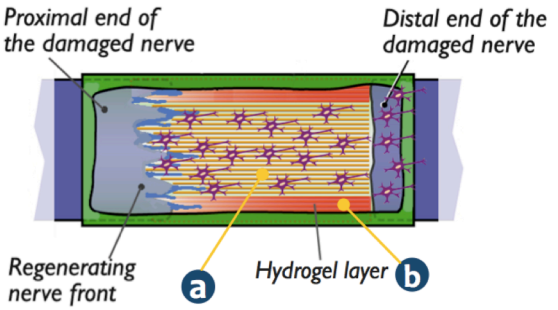

Fig. 1. Design of the nanofiber nerve guides incorporating the nanofiber guidance cues (a) and neurotrophic factor gradient loaded in the hydrogel layer (b) between the outer membrane and nanofibers.

Tasks 2a-2d were carried out as outlined below.

Optimizing Nanofiber Guidance (tasks 2a-2d)

These tasks were divided into specific tasks related to various components of optimization schemes.

In vitro fiber size dependent Schwann cell migration for optimization of NGCs

To optimize the configuration of the aligned fiber substrates in our nerve guidance conduits, we investigated the effects of fiber diameter on the migration kinetics of human primary Schwann cells. Such studies were highly important to determine the optimal fiber size for maximizing the speed of migration and directional guidance of migration, which were important factors for maximizing the rate of nerve regeneration through our nerve conduits and subsequently maximizing functional outcome in *in vivo* systems.

Improvements in our live-cell microscopy and MATLAB-based cell migration allowed for high-throughput cell tracking studies of dissociated Schwann cells migrating on a wide range of fiber sizes and configurations. Studies were conducted by culturing primary human Schwann cells with labeled nuclei on nano/microfiber-coated coverslips in 24 well plates, observing cell migration using live-cell imaging microscopy, and analyzing cell migration with our MATLAB-based programs.

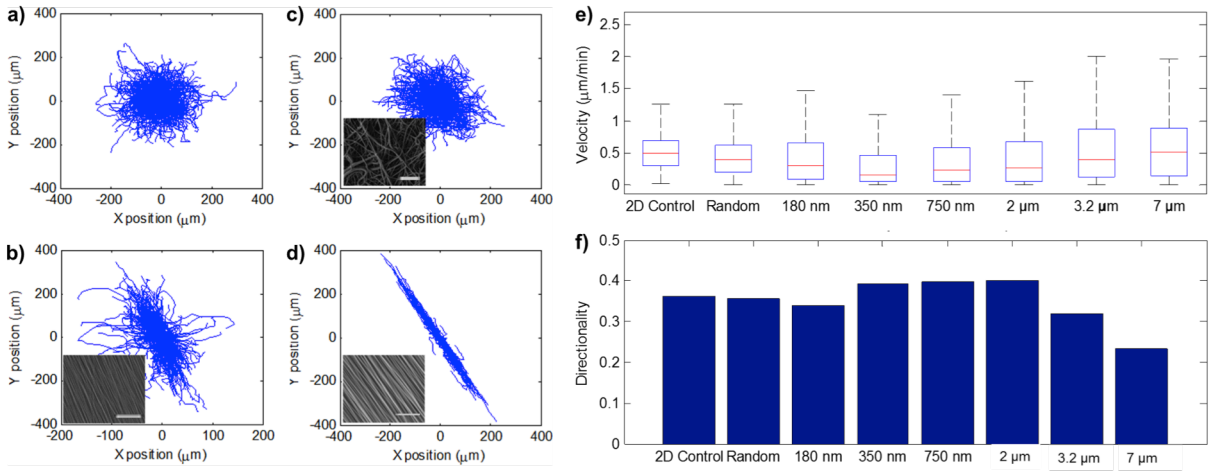

Fig. 2. Fiber size-dependent migration of human primary Schwann cells on polycaprolactone nano/micro-fibers. Migration pattern of cells on 2D control (a), random nanofibers (b), 180 nm aligned nanofibers (c), and 750 nm aligned nanofibers (D). Scale bars = 30 μm. Cell migration velocity (e) and migration persistence (f) of cells on varying substrates. For all figures, n > 450 cells for each group.

Our studies demonstrated that aligned fiber substrates provided enhanced topographical restriction of the migration of cells along a single axis (Fig. 2d) compared to 2-dimensional substrates (Fig. 2a) and random fiber substrates (Fig. 2b). We hypothesize that this topographical restriction is an important factor in the promotion of uniaxial migration of nerve outgrowth and will aid in faster peripheral nerve functional recovery where nerve outgrowth occurs along linear paths. For these single-cell studies, the

ability for aligned fibers to restrict the migration direction of cells was shown to be fiber-size dependent. Our results show that small nanofibers (180 nm, Fig. 2c) were less able to restrict cell migration direction than larger fibers (Fig. 2d), and cells were better able to migrate onto neighboring fibers. This transmigration appeared to become limited as fiber size increases, indicating a preference for the cells to migrate along a single fiber once fiber size becomes suitably large.

We also demonstrated that cell migration speed is dependent on fiber diameter, with cell migration rate increasing as fiber diameter increases (Fig. 2e). Based on our results, cells migrating on aligned fiber substrates with fiber diameters of less than 1 μm exhibited migration speeds lower than that of two-dimensional substrates. Fibers larger than 1 μm in diameter exhibited cell migration velocities that were comparable or faster than two-dimensional substrates. However, although the speed of migration was higher on larger fibers, cell migration direction fluctuated more on larger fibers, and thus they exhibited lower migration persistence compared to small fibers and 2D controls (Fig. 2f). This indicates that although the speed of migration is increased on larger fiber substrates, additional directional guidance cues, such as neurotrophic factor gradients, are necessary to improve the regenerative efficacy of fiber-based nerve guidance conduits.

In addition to single-cell live-cell tracking, we conducted multi-day spheroid cultures to investigate the bulk migration of cells in 3D in response to different topographical cues over extended periods of time. These cultures simulated the bulk migration of Schwann cells from a nerve stump following nerve injury. To conduct these studies, we first cultured GFP-transfected primary human Schwann cells into spheroids using the Microtissues agarose mold technique. We then seeded the spheroids onto nano/microfiber-coated coverslips, and sequentially imaged each spheroid after 24, 48, and 96 hours (Fig. 3). The use of spheroids allowed for simple qualitative and quantitative analysis of bulk cell migration from a localized point of origin. It was found that Schwann cells migrated further from the spheroid on all the aligned fiber groups as compared to either bare glass or randomly aligned nanofiber groups (Fig. 4). Cells were able to migrate at rates of approximately 1mm/day, which corresponds to ~0.7um/min. This rate is above the median rate seen in the live-cell tracking experiment.

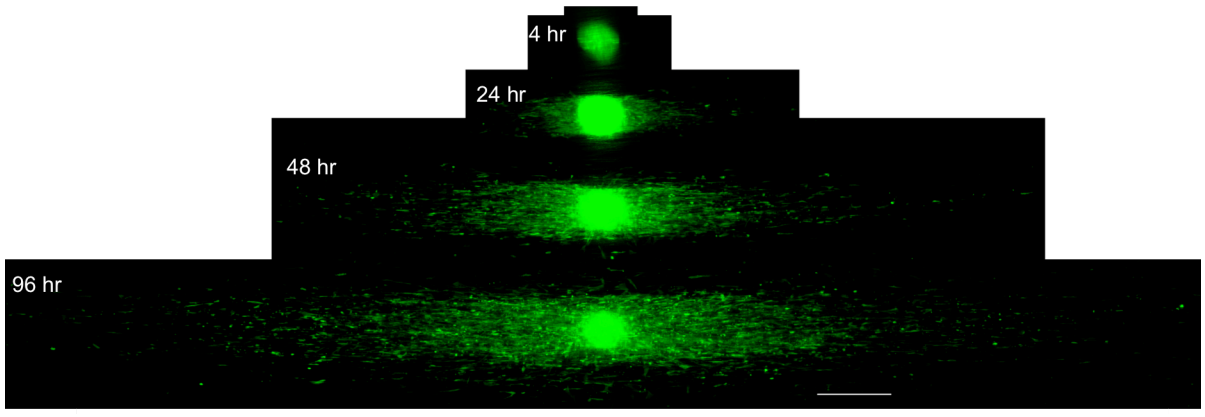

Fig. 3. GFP-transfected human primary Schwann cells migrating out from the same spheroid over time. The spheroid was seeded onto 1.4μm aligned nanofibers oriented horizontally in the image. The scale bar is 500μm

Interestingly, the migration rate from spheroids did not greatly vary with nano/microfiber size. We hypothesize that paracrine signaling or cell contact repulsion resulted in increased rates of cell migration in spheroids cultures compared to single-cell studies and minimized migration velocity differences between fiber groups. The primary difference between fiber groups was that smaller fiber sizes were less able to restrict trans-fiber cell migration, resulting in greater bi-axial migration and lower migration aspect ratio compared to the other aligned fiber groups (Fig. 5).

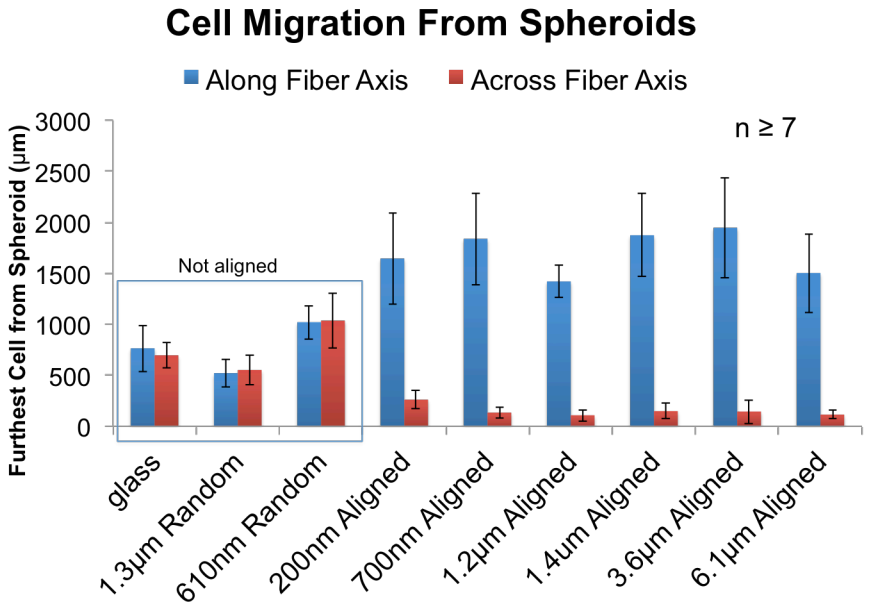

Fig. 4. Maximum cell migration of Schwann cells from edge of cell spheroid after 48 hours

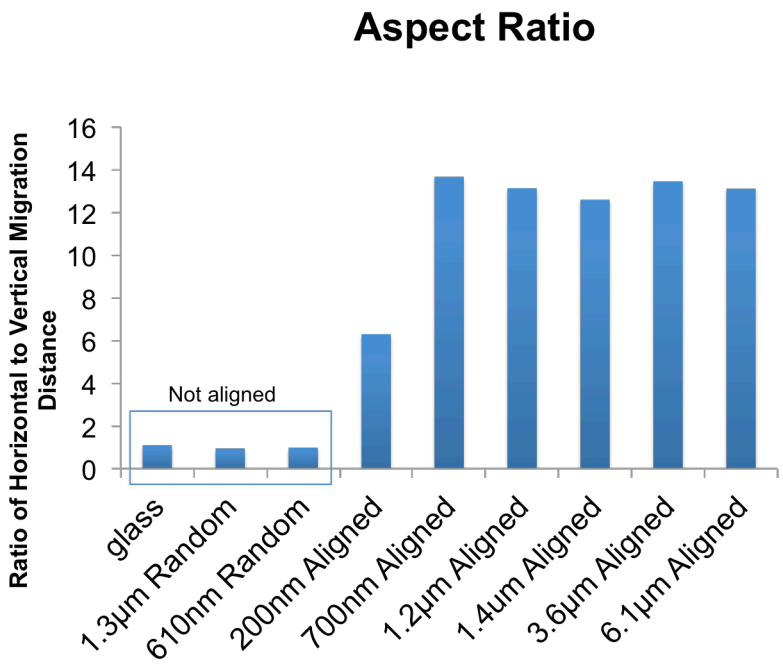

Fig. 5. Averaged aspect ratio of spheroid migration groups at 48hours. The aspect ratio is the horizontal migration distance divided by vertical distance travelled.

We also tested for cell attachment preferences for cells grown in 3D environments by casting a hyaluronic gel over the spheroids grown on the nano/microfibers. Gels were formed with sufficiently high porosity to allow cell migration, per other cell studies, and with sufficient laminin to allow cells to infiltrate the gel from the fiber surface. Nonetheless, the cells spread on the fibers instead of migrating into the hydrogel. Although cell migration occurred primarily on the fiber surface, the cell migration

was about half the rate of the migration compared to spheroids grown on fiber surfaces without an overlying hydrogel. These results demonstrate that incorporation of aligned fibers into a 3D environment can be an effective method of promoting uniaxial migration in environments comparable to that of a regenerating nerve. They also confirm the findings of the single-cell studies showing that additional directional cues, such as neurotropic gradients, are necessary to promote unidirectional migration of Schwann cells.

Neurotrophic factor gradient delivery for migration guidance of Schwann cells (task 2c/d)
In the past year, we have continued to investigate the effects of diffusible neurotrophic factor gradients on Schwann cell migration guidance. Much of our work has involved improving the efficacy of our cell tracking methodology, which we have now developed into a system capable of semi-automatic, simultaneous tracking of thousands of cells exposed to a variety of neurotrophic factor gradients. We developed a migration chamber setup with which we can simultaneously incorporate topographical and biochemical cues (Fig. 6a,b). A PDMS migration chamber with a controlled height is placed over the hydrogel/fiber construct (Fig. 6c) to limit the height of media and prolong the maintenance of neurotrophic factor gradients as they diffuse from the hydrogel into media. Cells are then pipetted into the chamber and their path of migration is observed using a live-cell imaging microscope (Fig. 6d). Utilizing this migration chamber setup we developed, we have a platform with which we can control both biochemical and topographical cues and monitor thousands of cells in real-time as they respond to the provided cues (Fig. 6 e-g). This platform gives us a powerful tool for determining which cues are most effective in enhancing neural cell migration and promoting directional migration guidance.

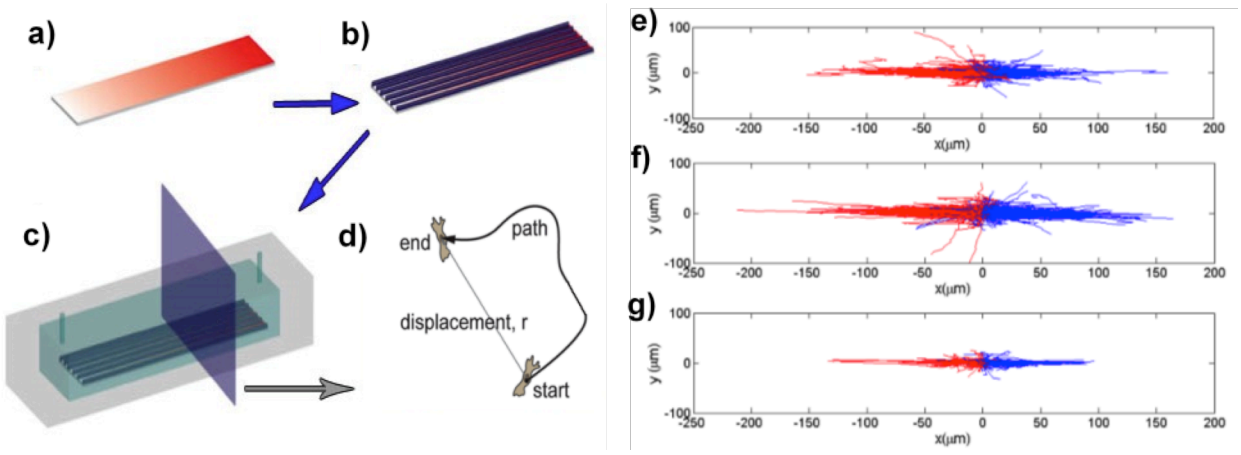

Fig. 6. *In vitro* live-cell migration chamber allowing for high-throughput cell tracking. Gradient hydrogels with controlled gradient characteristics are generated (a) and aligned nanofibers are placed over the hydrogel (b). PDMS migration chamber is placed over the hydrogel/fiber construct (c) and cell migration is tracked from point of origin and along the entire trajectory (d). This method was used to monitor cell migration of primary human Schwann cells exposed to 0-1 μg/mL (e), 0-10 μg/mL (f), and 0-20 μg/mL (g) GDNF gradients, simultaneously tracking over 1000 cells per sample.

As our primary neurotrophic factor of interest for *in vivo* studies has been glial derived neurotrophic factor (GDNF), many of our gradient-based studies have investigated the effects of varying GDNF gradient characteristics on Schwann cell migration kinetics and directional bias. Specifically, we were interested in determining how GDNF gradient concentration range affected the migration velocity and directional bias of Schwann cells, and found that markedly different cellular responses occur when increasing the concentration range of the GDNF gradients. Using our newly-developed migration chamber platform, we were able to show that Schwann cell migration velocity increased significantly

as the concentration range of GDNF gradient increased (Fig. 7a. This indicated that GDNF provided a concentration-dependent pro-migratory cue. However, as GDNF gradient concentration range increased, directional migration bias decreases (Fig. 7b), demonstrating that when gradient concentrations are too high, cells are less able to sense the direction of the gradient decreases. These results demonstrate the importance of designing gradients that balance pro-migratory cues with sufficient directional guidance, considerations that were important for the incorporation of gradients into *in vivo* nerve guides.

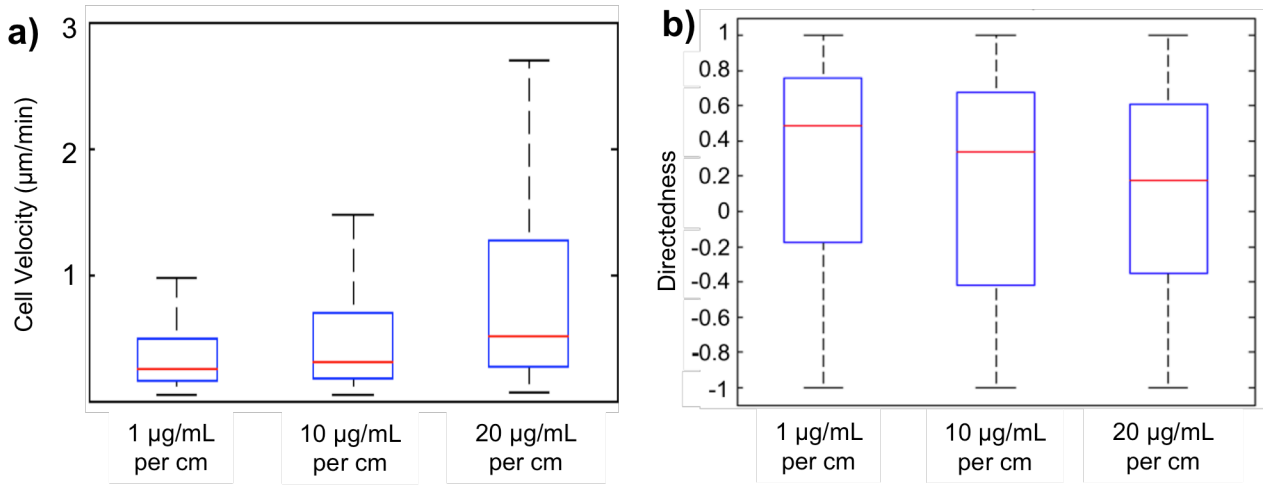

Fig. 7. Primary human Schwann cell migration in response to varying gradients of GDNF. Cell migration velocity of cells exposed to GDNF gradients with increasing concentration ranges (a). Directedness, or migrational bias, of migration with a value of 1 indicating cells migrating perfectly in the direction of gradient, 0 being non-biased migration, and -1 indicating cells migrating in opposite direction of gradient (b). Cell number > 1000 for analysis.

In vivo nerve guide design (tasks 2a-d)

We have now tested 20 nerve guide designs in 124 rats, as enumerated in table 1. By varying hydrogel properties, nanofiber properties, gradient properties, and overall nerve guide configuration, we have dramatically improved the efficacy of regeneration of our nerve guides.

The 2012 annual report details the results of groups 1-8 and the plans for groups 9-13, but here is a brief synopsis. We initially hypothesized that we could improve nerve regeneration by amplifying the signal from the aligned fiber layer by presenting a larger surface area to the regenerating nerve. We accomplished this by replacing the single, luminal layer of aligned fibers with a spirally-wound set of aligned fiber layers. Our first groups (#3-#8) used crosslinked gelatin films as the backing material, with controls of the original single luminal layer (group 2) and a spiral with no aligned fibers (group 1). While the spiral nanofiber groups had better regeneration than the controls, the results indicated some problems with the design. The gelatin films degraded much slower than anticipated, while also swelling more than desired. The swelling of the gelatin caused it to compact outward, packing the layers too tightly to allow nerve fascicles to interact with the individual layers. The regenerating tissue was essentially only seeing one layer of the aligned fibers, which obviated the point of the spiral design. The initial groups also showed that GDNF inclusion into the nerve guide could improve growth of nerve into the nerve guide, but was unable to promote regeneration across the entire nerve gap due to the lack of directional guidance.

The second spiral design, tested via groups 9-13 in March 2013, sought to preserve the idea of using a spiral to increase the aligned-fiber surface area, but to replace the ineffective gelatin backing material. Modifying the nanofiber spinning procedure to produce robust sheets of purely aligned

nanofibers allowed us to replace the gelatin with weaker options that can be formed on or around pre-spun nanofibers. We chose fibrin gel as a replacement for gelatin since it already plays a role in the body's response to a nerve injury.

	Groups	# rats	Fiber Distribution	Aligned Fiber Density	Fiber Diameter (nm)	Composition % of Gelatin (PCL is Remainder)	GDNF Loading (ng/tube)	Implantation Date
	1	8	spiral no fiber				0	
	2	8	Single Layer	medium	760	0	0	
Tier 1	3	8	Spiral	medium	760	0	0	Jun-12
	4	8	Spiral	high	760	0	0	
	5	8	Spiral	low	760	0	0	
	6	6	Spiral	medium	760	0	20	
	7	6	Spiral	medium	760	0	200	Oct-12
Tier 2	8	6	Spiral	medium	760	0	2000	
	9	6	Fibrin Spiral	medium	**1200**	0	600	3/5/13
	10	6	**Fibrin Spiral**	medium	760	0	600	3/7/13
Tier 3	**11**	6	Fibrin Spiral	medium	**400**	0	600	3/8/13
	12	6	Fibrin Spiral	medium	760	**90**	600	3/12/13
	13	6	Fibrin Spiral	medium	760	**80**	600	3/13/13
	14	4	**Microfibers**	medium		0	~600	5/6/13
Tier 4	15	4	**Fringe Spiral**	medium	1200	0	~600	5/6/13
	16	4	**"S" open design**	medium	1200	0	~600	5/7/13
	17	4	S design	medium	1200	0	Uniform	6/27/13
Tier 5	18	4	S Design	medium	1200	0	Shallow Gradient 60-180	6/28/13
	19	8	S Design	medium	1200	0	Steep Gradient 1-240	6/28/13
	20	8	1 layer	medium	1200	0	Steep Gradient 1-240	10/8/13

Table 1. Overview of nerve guide designs tested to date.

Results of groups 9-13:

Histology Results

The change in design from gelatin films to fibrin gels was successful, in that the layers of nanofibers were better distributed within the nerve luminal space. Also, there was a great improvement in the functional electrophysiological results over the previous tier. However, in many of the samples, the nerve bundles were not interspersed amidst the nanofibers and fibrin in the middle of the conduit, but rather pressed in between the outer conduit wall and the PVA-collagen hydrogel, never even seeing the nanofibers (Figures 8 and 9)

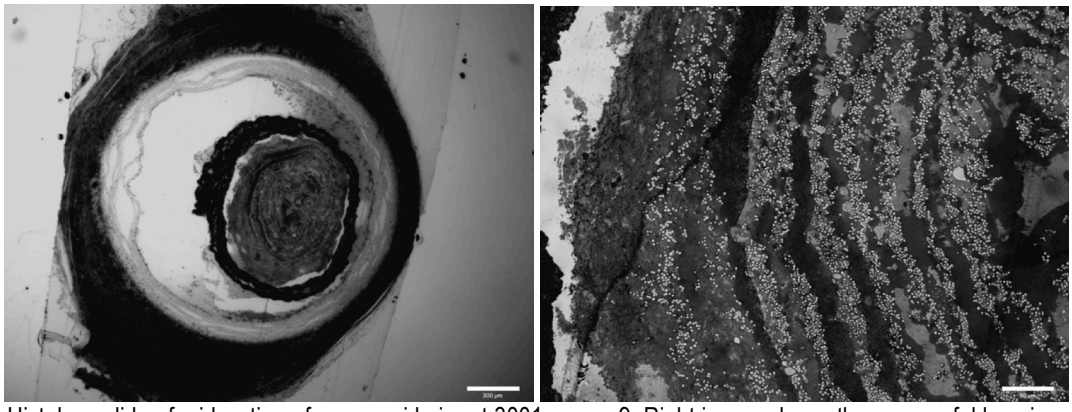

Fig. 8. Histology slide of midsection of nerve guide in rat 3001, group 9. Right image shows the successful layering of nanofibers in a theoretically cell permeable fibrin gel. Left scalebar is 300 µm, right scalebar is 30 µm.

Our hypothesis is that the fibrin layer was too dense right at the interface of the proximal nerve stump, and the regenerating nerve took the path of least resistance, which took it to the lumen periphery. Additionally, the growth factor, GDNF, was being released by the hydrogel in the periphery of the

lumen, so that it may be acting like a chemo-attractant for the nerve tissue towards the periphery. The degradable nanofibers were successfully degraded by the 8 week timepoint, no longer being visible in the histology slides. However, they did not seem to produce improved regeneration in either the histology or electrophysiology data.

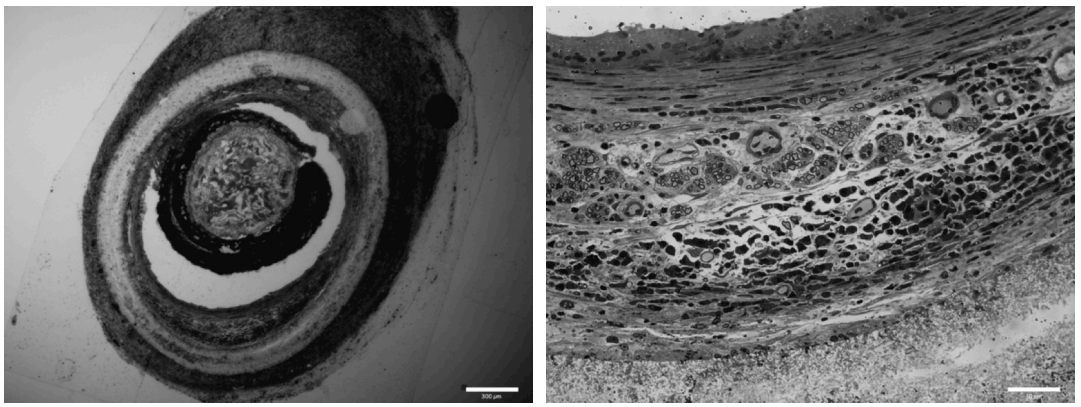

Fig. 9. Histology slide of midsection of rat 3009, group 10. Right: note the presence of axons in the cell field along the inner wall of nerve guide Left scalebar is 300 µm, right scalebar is 30 µm.

Electrophysiology results

While a motor signal could be detected in some of the subjects in the foot muscles, the data was too inconsistent to differentiate the groups. The left ankle motor data, however, was detectable in all the rats and had pronounced differences between rats. Since there was less than expected interactions between the host tissue and the nanofibers, the ideal nanofiber characteristics were still not clear.

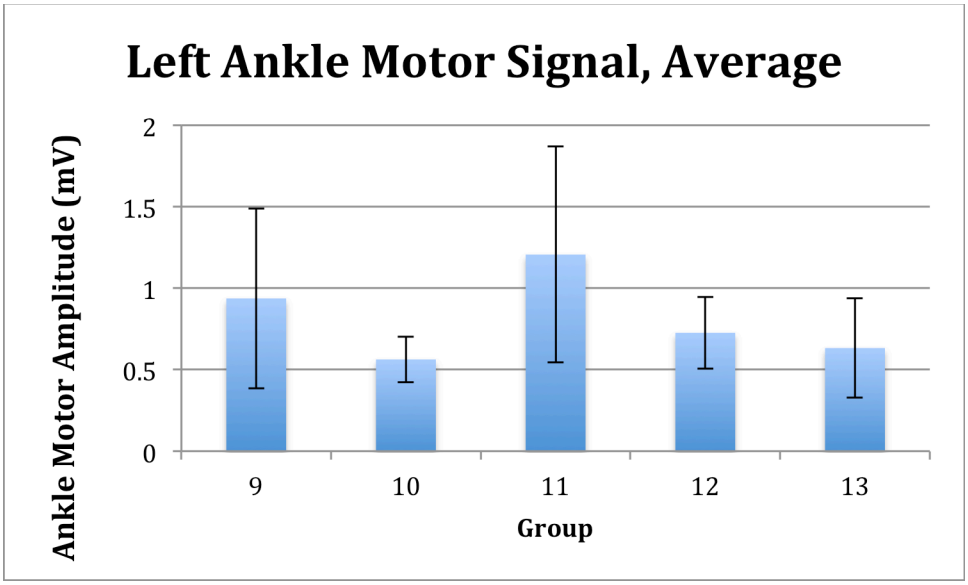

Fig. 10. Electrophysiology data for groups 9-13. The amplitude of the motor signal sent from a needle electrode at the sciatic notch is detected via an electrode inserted into the tibial nerve in the left (injured) ankle.

Groups 14-16: Design and Results

Tier 4, groups 14-16, was designed to explore ways to overcome the problems of the fibrin-spiral design. The nerve guides were designed to have more open area for easy infiltration by the proximal nerve stump. The first group, 14, replaced the fibrin-based nanofiber spiral with a bundle of hydrogel

microfibers developed by collaborators in the Mao lab. The next group, 15, is similar to the previous fibrin-spiral design, but without the fibrin in the 1mm closest to the proximal nerve stump, just the loose aligned fibers, to allow the nerve cells to attach to the loose fibers before seeing a mechanical barrier. The final group, 16, is modeled after the “S” configuration used by Andres Hurtado et al in their spinal cord conduit (Figure 11). Here, the majority of the luminal space is open, with a double-sided film of aligned nanofibers to provide topographical guidance. The backing film of the “S” design was made from partially-melting together a mesh of random PCL nanofibers, which will ideally provide structural support without compromising diffusion. This design was also inspired by the histological results of groups 1-8. While having poor regeneration overall, the areas of regenerating nerve tissue could often be found in sections of the nerve guide where the gelatin sheet had folded back on itself, leaving an oval space at least 100microns across. The “S” design intentionally incorporates similar folds. Additionally, the central curve of the S-shape has some inherent elasticity, and can provide additional strength in resisting compressive stresses that the nerve guide might endure *in vivo*, further protecting the regenerating nerve from impingement due to surrounding tissue or movement. All three groups have replaced the PVA-collagen hydrogel with alginate-based microfibers for releasing the GDNF. This allows the growth-factor release to be near the nanofibers. Since this tier had been designed to optimize the interface between the proximal stump and our topographical guidance, our experiment was designed to best test this component, utilizing a shorter 7mm gap between nerve ends, a shorter endpoint of 4 weeks, and longitudinal sectioning to best capture that interface.

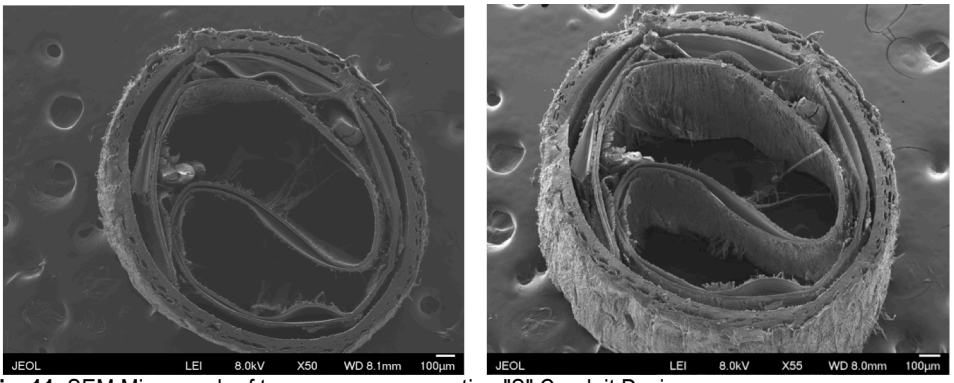

Fig. 11. SEM Micrograph of transverse cross-section "S" Conduit Design.

Histology results, groups 14-16

The histology results were difficult to interpret. The timepoint was short at 4 weeks, so only limited regeneration was to be expected. Many of the samples had less cross-sectional area than expected from the electrophysiology results. The microfibers in group 14 seem to have degraded away by this 4 week timepoint. The group 15 samples still had discernable nanofibers, yet lost the obvious spiral structure seen in groups 9-13. This may be due to the elimination of the PVA-collagen hydrogel layer that kept the layers bound up. There were interactions between the cells and the nanofibers, but not particularly with the axons.

Rat 2960, however, had a large area dense with axons in one of the loops formed by the S-design. This rat also had a large amplitude in the ankle motor signal. This was the single best result from this tier of nerve guides, so we used this as the basis for the next tier.

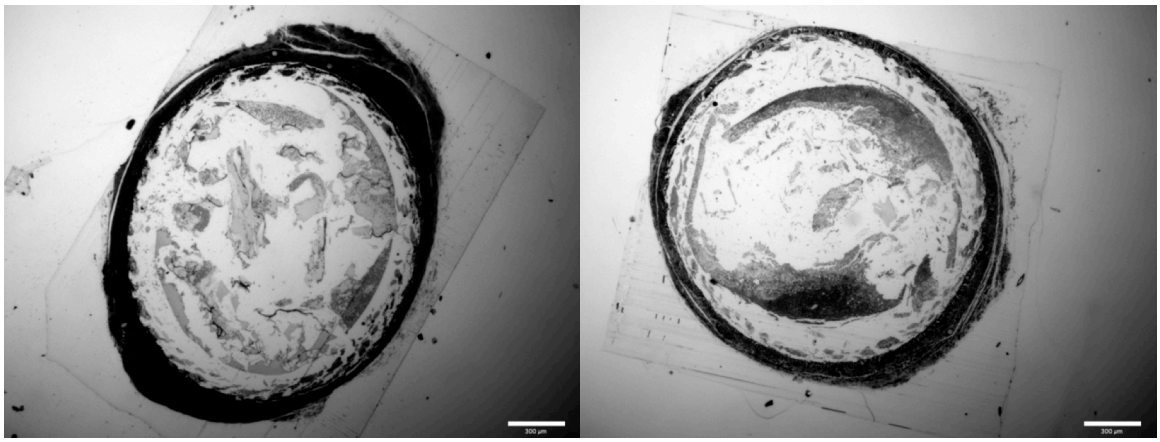

Fig. 12. Histology slides of (left) rat 2953, group 14, and (right) rat 2956, group 15. Scale bars are 300 µm.

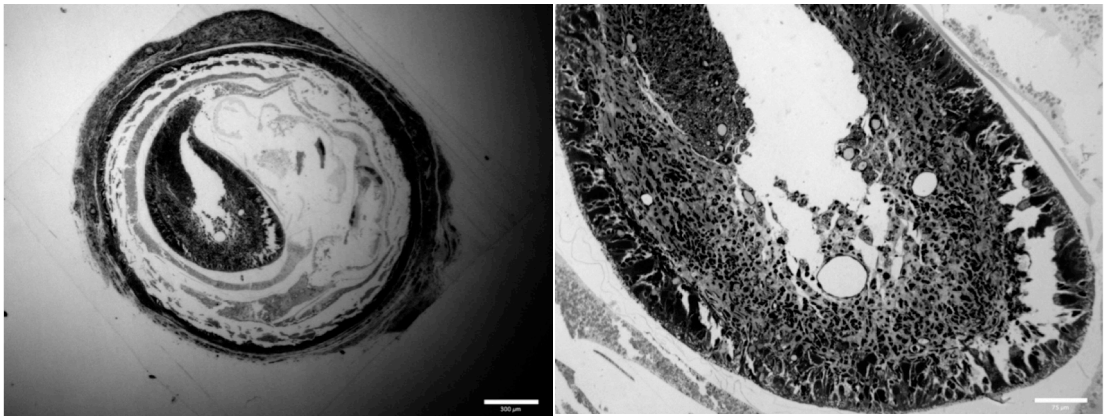

Fig. 13. Histology slide of Rat 2960, group 16. Left scale bar is 300 µm, right scale bar is 75 µm

Electrophysiology results, groups 14-16

The rats showed varied results in the electrophysiology functional assay (Figure 14). Some of the rats in each group had low amplitudes (~0.2mV) in the ankle, while others were much higher, 0.6-1mV. From this result alone, it is hard to judge the best design. With the histology results, however, group 16 had the best overall example of regeneration.

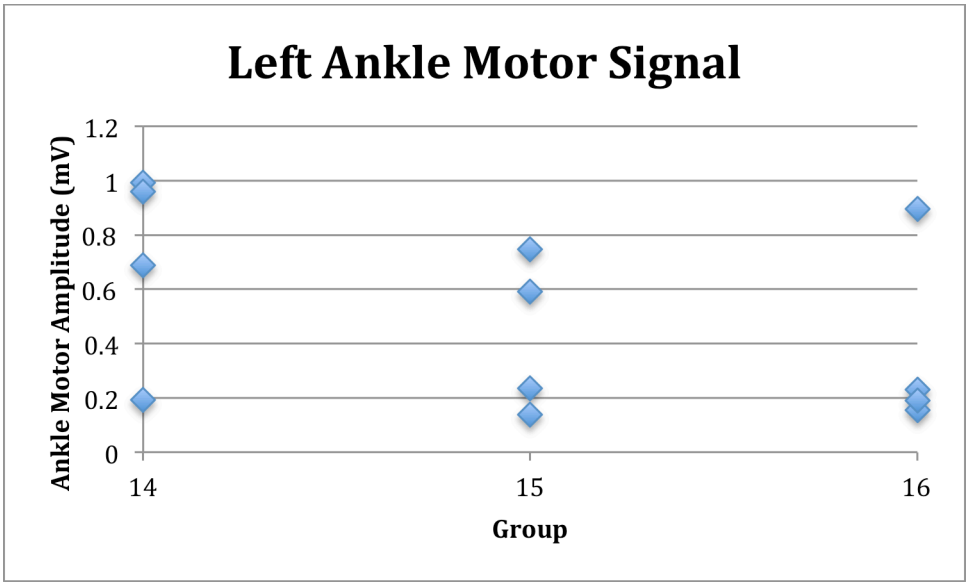

Fig. 14. Amplitude of the motor signal in the treatment leg with electrodes placed at the sciatic notch and into the tibial nerve at the ankle.

Groups 17-19: Testing Growth Factor Gradients in vivo

We have completed an additional tier of nerve guides to test whether a gradient of GDNF could improve the nerve regeneration. Based upon the positive result of the group 16 “S” design, we used this as the platform for testing the gradients in vivo. There were three groups to test the GDNF release, all with the same total loading of GDNF, 600ng/guide. In group 17, the GDNF was uniformly loaded (no gradient). In group 18, the GDNF was loaded as a shallow gradient, from 60ng/mL to 180ng/mL loading the the gelatin. Group 19 had a steeper gradient, from 1 to 240ng/mL GDNF loading within the gelatin. This configuration improves upon the previous group 16 “S” design in two ways. The backing film for the aligned nanofibers in group 16 was nonporous, while in groups 17-19 it is heat-treated less, so as to have adequate strength yet remain fibrous and thus porous. Secondly, in group 16, the GDNF was released from alginate microfibers. These may have eluted out the GDNF quicker than desired, over only a few days. The crosslinked-gelatin films used in gradient production should release the growth factor over a longer timeframe, and with the porous backing, should disperse more evenly throughout the nerve guide.

Electrophysiology and Histology Results

The electrophysiology and histology datasets were very promising. For electrophysiology (Figure 15), the uniform GDNF loading of group 17 showed similar results as group 16 (Similar design, but with GDNF release from alginate microfibers), with some low motor signal amplitudes and some higher amplitudes in the ankle. The shallow gradient group, 18, had poor signal amplitudes. The steep gradient group, 19, however, had two rats with motor signals with much higher amplitude than any of the other rats with this 4week timepoint. This shows that the steep gradient may improve the regeneration. This may show that a steep gradient is necessary to provide a guidance cue to the regenerating tissue. The poor signal in some of the rats in groups 17-19 may be due to axonal trapping caused by having a high concentration of GDNF in the distal end of the conduit, which would discourage axonal reinnervation of the distal stump. This trapping may diminish as the GDNF levels decrease at longer timepoints than 4 weeks.

The histology results were even more impressive (Figures 16 and 17). In almost every sample there was dense cell fields with axons in both main spaces created by the S shape, as well as often between the “S” wrap and the outer wall. The regenerating cells interacted more with the aligned nanofiber layers, with nanofibers being pulled well within the cell field as seen in the figure below. The advantages of a steep versus a shallow gradient are not obvious from the histology data alone.

Almost every single sample had larger cell fields than even the best group 16 sample (only rat 2965 looked worse than the best sample from group 16, #2960). This illustrates the improvement made by making the backing walls more porous and allowing for better release of GDNF into the lumens.

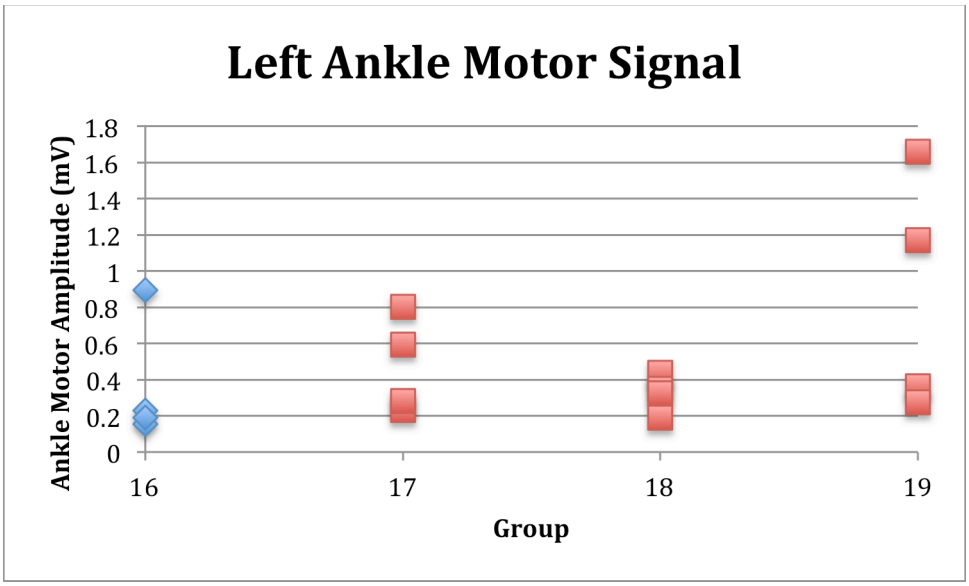

Fig. 15. Amplitude of the motor signal in the treatment leg with electrodes placed at the sciatic notch and into the tibial nerve at the ankle.

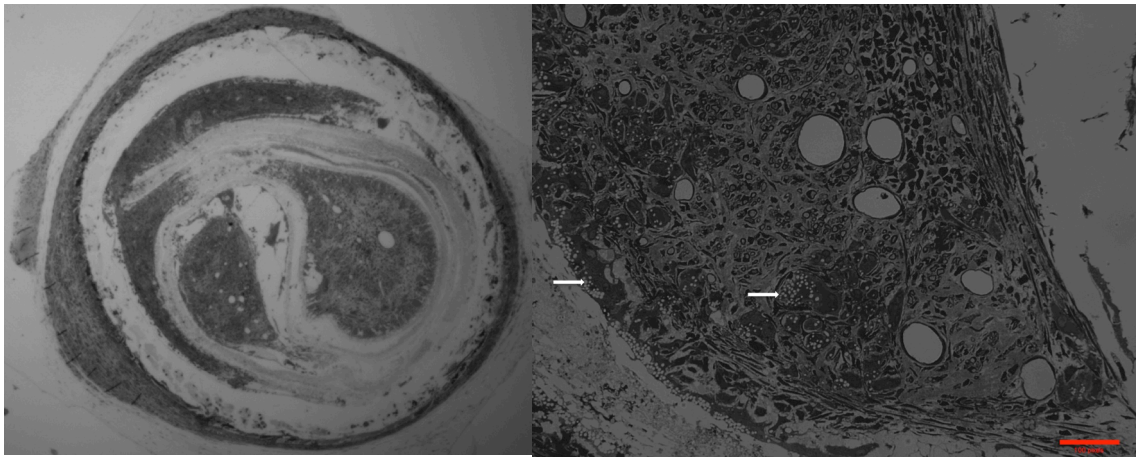

Fig. 16. Transverse cross-section of midpoint of group 18 nerve guide at the 4week timepoint. This nerve guide had the "S": design with a shallow gradient. Right: increased magnification of the left fascicle. The white arrows indicate aligned fibers both at their original position on the backing films well as being enveloped well within the cell field.

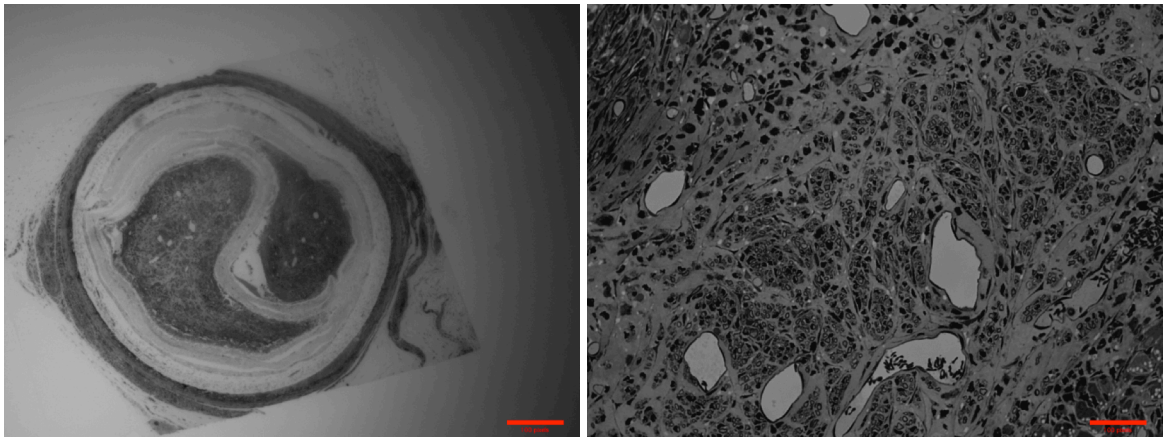

Fig. 27. Transverse cross-section of midpoint of group 19 nerve guide at the 4week timepoint. This nerve guide had the "S": design with a steep gradient. Right: increased magnification of the left fascicle.

Final Composition of the Optimized NGC

Based upon the results of the gradient tier of nerve guides, we believe that we have determined the optimal design for the upcoming dog study. To verify the results, we have run one final set of in vivo experiments in October, 2013. We have implanted 4 more rats with group 19 nerve guides ("S" design with steep gradient of GDNF) to have 8 total rats in the group to prove reproducibility. We have also implanted 8 rats with group 20 nerve guides, with the steep GDNF gradient, but without the "S" shape, with only a single, peripheral layer of aligned fibers, to determine the contributing effects of the S-shape versus gradient. We still are waiting for the histological results. Once we have this data analyzed, we will know the optimal nerve guide configuration to use in the upcoming dog study to achieve the best possible regeneration.

Regulatory approval for the dog studies (Task 2e)

We have obtained both IACUC and ACURO approvals for the large animal validation studies.

Large Animal Validation Studies (Task 3)

This is the task that was delayed due to delays in optimization of the NGCs. In Year 3 we completed our IACUC and ACURO approvals and have carried out the nerve repairs in one of the control groups. This control group consisted of autologous nerve transfer to mimic the current state-of-the-art in human nerve repairs using sural nerve grafts. These surgeries were carried out in December 2013 and early January 2014.

We have the additional animals and expect to carry out the second control group using NeuraGen tubes made out of hollow collagen tubes. These tubes are currently used in patients undergoing short distance nerve repairs. They will serve as controls as the current state-of-the-art biodegradable nerve conduits.

This month we'll be completing the histological analysis in Group 20 and will start manufacturing the nerve guides to be used in the dog studies (Task 3a) and start doing the nerve repairs in the remaining dogs (Task 3b).

Key Research Accomplishments:
Refinement of the nanofiber NGCs:

- Increased nanofiber surface area
- Fine-tuning of nanofiber degradation rate
- Gradient loading of neurotrophic factors
- Evaluation of Schwann cell invasion and motility on nanofiber NGCs

Validation studies in the rat sciatic nerve regeneration model

- Tested the role of increased nanofiber surface area (spiral design)
- Tested the role of gelatin hydrogel with GDNF loading
- Tested the role of nanofiber density
- Tested the role of gradient neurotrophic factor loading
- Tested the optimum combination of NGCs

Reportable Outcomes

Manuscripts Published
Krick K, Tammia M, Martin R, Höke A, Mao HQ. Signaling cue presentation and cell delivery to promote nerve regeneration. Current Opinions in Biotechnology, 22(5): 741-746 (2011).

(Not directly supported by this grant, but very much relevant to overall aims of enhancing peripheral nerve regeneration using topographical cues:
Ren YJ, Zhang S, Mi R, Liu Q, Zeng X, Rao M, Hoke A, Mao HQ. Enhanced differentiation of human neural crest stem cells towards the Schwann cell lineage by aligned electrospun fiber matrix. Acta Biomater. 2013 Aug;9(8):7727-36. PMID: 23628775

Jiang X, Mi R, Hoke A, Chew SY. Nanofibrous nerve conduit-enhanced peripheral nerve regeneration. J Tissue Eng Regen Med. 2012 Jun 15. PMID: 22700359)

Scientific Presentations

Krick KD, Khademhosseini A, Höke A* and Mao HQ*. Neurotrophic Factor Gradient Delivery for Migration Guidance of Schwann Cells, Poster Presentation at the BMES Annual Meeting, Seattle, September 2013

Conclusion:
We have evaluated the role of multiple variables relevant to design and optimization of the nanofiber NGCs and tested them in the rat studies. We have obtained IACUC and ACURO approval for the large animal validation study and have started those experiments. We plan to complete the large animal validation studies in this no-cost extension year and report our final outcomes at the end of the year.

References:
None

Appendices:
None